Biochimica et Biophysica Acta 1802 (2010) 1193–1202

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Ménage à trois: Aldosterone, sodium and nitric oxide in vascular endothelium

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ARTICLE INFO

Article history:
Received 17 November 2009
Received in revised form 10 March 2010
Accepted 11 March 2010
Available online 17 March 2010

Keywords:
Aldosterone
Nitric oxide
ENaC
Endothelia
Sodium

ABSTRACT

Aldosterone, a mineralocorticoid hormone mainly synthesized in the adrenal cortex, has been recognized to be a regulator of cell mechanics. Recent data from a number of laboratories implicate that, besides kidney, the cardiovascular system is an important target for aldosterone. In the endothelium, it promotes the expression of epithelial sodium channels (ENaC) and modifies the morphology of cells in terms of mechanical stiffness, surface area and volume. Additionally, it renders the cells highly sensitive to small changes in extracellular sodium and potassium. In this context, the time course of aldosterone action is pivotal. In the fast (seconds to minutes), non-genomic signalling pathway vascular endothelial cells respond to aldosterone with transient swelling, softening and insertion of ENaC in the apical plasma membrane. In parallel, nitric oxide (NO) is released from the cells. In the long-term (hours), aldosterone has opposite effects: The mechanical stiffness increases, the cells shrink and NO production decreases. This leads to the conclusion that both the physiology and pathophysiology of aldosterone action in the vascular endothelium are closely related. Aldosterone, at concentrations in the physiological range and over limited time periods can stabilize blood pressure and regulate tissue perfusion while chronically high concentrations of this hormone over extended time periods impair sodium homeostasis promoting endothelial dysfunction and the development of tissue fibrosis.

1. Aldosterone, a steroid which affects the kidney and the blood vessels

Aldosterone, a mineralocorticoid hormone mainly synthesized in the zona glomerulosa of the suprarenal glands, plays a pivotal role in the control of body fluids and blood pressure. Its concentration in the plasma of healthy humans varies over a wide range, between 0.05 and 0.5 nM. A major stimulus for its secretion is a decrease in extracellular fluid and/or a fall in blood pressure that triggers a cascade of enzyme activation steps, which, within seconds, lead to the release of the hormone from the suprarenal glands into the blood. The classical targets for this hormone are the principal cells of the renal collecting duct [1] in which aldosterone binds to intracellular mineralocorticoid receptors which enhances NaCl reabsorption. In a close interaction with other mechanisms (e.g., renal retention of water by the antidiuretic hormone), NaCl and water are retained and the blood pressure returns to normal.

It is worth looking at 'time scales and quantities' in order to evaluate the efficiency of renal aldosterone action in the control of blood pressure. Though chemically a steroid, the hormone is able to trigger fast responses in kidney cells [2–4]. Aldosterone elicits a number of cellular responses in seconds to minutes that finally lead to increased sodium chloride transport. As a rather conservative estimate, aldosterone-triggered salt retention in the renal collecting ducts starts about 30 min after the hormone is released from the suprarenal glands and continues as long as plasma aldosterone concentration remains raised. It is unlikely, however, that such a mechanism retains a significant amount of 'fluid volume' within a reasonably short time. For example, if all the NaCl in food is retained, then, with an average salt consumption of 8–12 g/day, the kidneys can only retain about 1 l of isotonic fluid in 24 h (150 mM NaCl = about 9 g/l) to increase blood pressure by volume expansion. If the adult human body is composed of about 20 l of extracellular fluid, i.e., the compartment where the retained salt and water will remain initially, then we must conclude that aldosterone cannot restore the blood pressure to normal within minutes or hours but only within days.

There is accumulating evidence that aldosterone acts on target cells of the cardiovascular system [5–9], particularly, the vascular endothelium [10,11]. Endothelial cells express mineralocorticoid receptors and functionally respond to the hormone [12–14]. In vitro, a prolonged exposure to aldosterone changes their morphology considerably and cells derived from different tissues grow in size and mechanically stiffen [11,15–17].

It has become increasingly evident that the mechanical stiffness of a cell is fundamental for it to function properly. Stiffness is important for cell motility, division, tissue organization, and cellular responses to biochemical and biophysical signals [18]. It is altered in

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doi:10.1016/j.bbadis.2010.03.006

pathological states, for instance the stiffness of cancer cells is reduced [19] but it is increased in chondrocytes in arthritis [20], airway smooth muscle cells in bronchial asthma [21] and ischemic cardiac muscle [22]. Endothelial cells have a vulnerability to mechanical shear forces that is likely to be increased when cells stiffen [23]. Furthermore, in terms of endothelial cell stiffness, aldosterone sensitizes the vascular endothelial cells to changes in plasma sodium [24] while it renders them rather insensitive to changes in plasma potassium [25].

2. Short-term action of aldosterone on the endothelium

A regulatory cell volume decrease after the acute application of aldosterone has been identified in vascular endothelium [26,27]. Interestingly, it has been shown that the aldosterone-triggered acute cell volume change is paralleled by a mirror-like change in cell stiffness (Fig. 1). In the initial phase of the hormone response (within 20 min), the cells swell and soften but after reaching a maximum, cell volume returns to the original volume while the cells stiffen. On returning to a steady state (aldosterone still present!), the change in cell volume is inconspicuous while the increase in cell stiffness persists. The increased stiffness indicates that mechanical forces are involved in mechanisms of cell shrinkage to its original volume. In parallel, cells contract [27]. The physiological response of a contracted endothelial cell to certain stimuli is likely to be abnormal. Vascular endothelium exposed to a raised concentration of aldosterone decreases its synthesis and release of nitric oxide [9,28,29]. This will have a major impact upon blood vessel function for NO is a key regulator of vascular tone. Since aldosterone stiffens endothelial cells it is probable that it is this increased stiffness which reduces NO release. This assumption is based upon the evidence that a pulsating flow of blood exerts shear forces at the endothelial cell wall, and the proposal that when such a cell becomes stiffer it will be more difficult to deform and thus reduce its synthesis and release of NO [30].

3. Long-term action of aldosterone on the endothelium

One of the initial responses of an endothelial cell to aldosterone is an increase in size [26]. It is probable that this increase is due to an increased uptake of salt and water mediated by a rise in apically expressed endothelial sodium channels [12]. This suggestion is strongly supported by the inhibitory action of amiloride, a sodium channel blocker used in mechanical measurements at single cell level. It is interesting that some recent observations indicate that such increases in cell volume may not be accompanied by an increase in cell pressure. Estrogens, another class of steroid hormones, swell endothelial cells even more than aldosterone but, at the same substantially decreases cell stiffness [17]. Such findings question whether aldosterone does indeed change the water content of a target cell rather than its solid mass.

This question was addressed by measuring cell water and solid cell mass at single cell level [23]. Atomic force microscopy is the most suitable method for such an approach, for several reasons: (i) Any structures with heights in the range of up to 12 µm, adherent to a solid surface, can be imaged at three dimensions and volumes quantified. (ii) Images can be obtained in fluid and in air. (iii) Fixed cells can be used so that the organic contents of a cell remain in place while water can be removed if desired. (iv) Surface coating that may influence the quantitative data is not necessary.

Surprisingly, aldosterone did not increase cell water. On the contrary, after 3 days there was a substantial increase in solid cell mass and a diminution of cell water (Fig. 2). It is possible that the sequence of events, triggered by aldosterone, is as follows: Upon application of the hormone there is a fast non-genomic response. This may comprise an influx of inorganic ions across the plasma membrane [2,31,32]. It is possible that even at this early stage of mineralocorticoid action, classical intracellular receptors may be involved [33]. The endothelial cell swelling observed as early as 5 min after application of the steroid, can be completely prevented by the aldosterone receptor blocker spironolactone [26]. The fact that the epithelial sodium channel blocker amiloride is also able to prevent swelling strongly indicates that sodium influx is closely related to the volume increase. As mentioned above, some 20 min later the volume of the endothelial cells decreases to normal [26]. Studies in oocytes suggest that within this first 20 min of aldosterone exposure the cell's genome is already activated, which leads to export extrusion of mRNA from the cell nucleus [34]. This suggests that the whole process of de novo protein synthesis has been launched. It is not surprising therefore, that, when aldosterone exposure is maintained over many days, the cells grow in size (Fig. 3). It emphasizes aldosterone's proliferative character. Furthermore, endothelium exposed to aldosterone shows 'gap formation' between cells. In the light of a recent study showing that aldosterone does not significantly change paracellular permeability [14], it is likely that such cells, surrounded by 'gaps' have entered apoptosis which can be blocked by spironolactone [35].

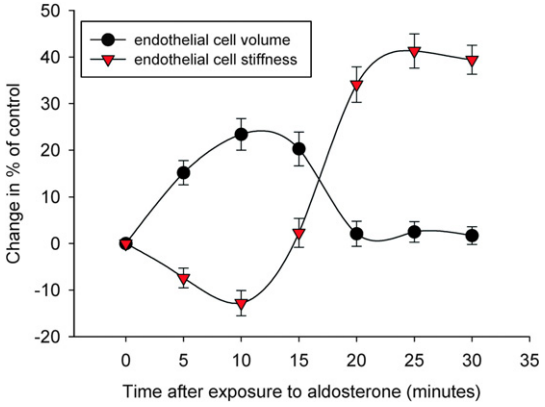

Fig. 1. Cell volume and cell stiffness, obtained in human endothelial cells after acute exposure to aldosterone. The transient volume increase is paralleled by a transient decrease in cell stiffness. While cell volume normalizes after some minutes, cell stiffness rises and remains increased as long as aldosterone is present (mean data of 10 measurements with 7–10 cells each are given). Cells were incubated in 100 nM aldosterone, 1 µM spironolactone (modified after [27]).

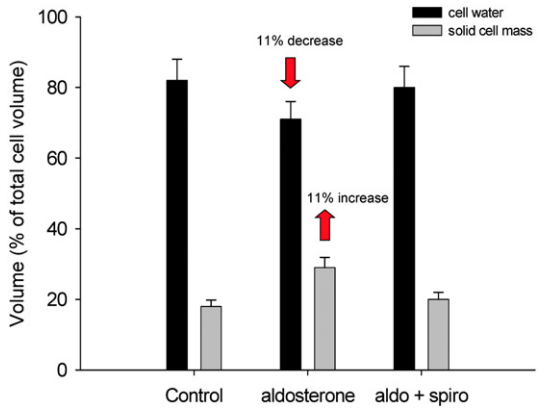

Fig. 2. Cell water and solid cell mass expressed in percent of total cell volume of individual human umbilical venous endothelial cells. Human endothelial cells were grown for 3 days either in the absence (control) or presence of 1 nM aldosterone alone and aldosterone (1 nM) + spironolactone (10 nM) (aldo + spiro). Please note that the sum of cell water and the solid cell mass amounts to total cell volume (modified after [23]).

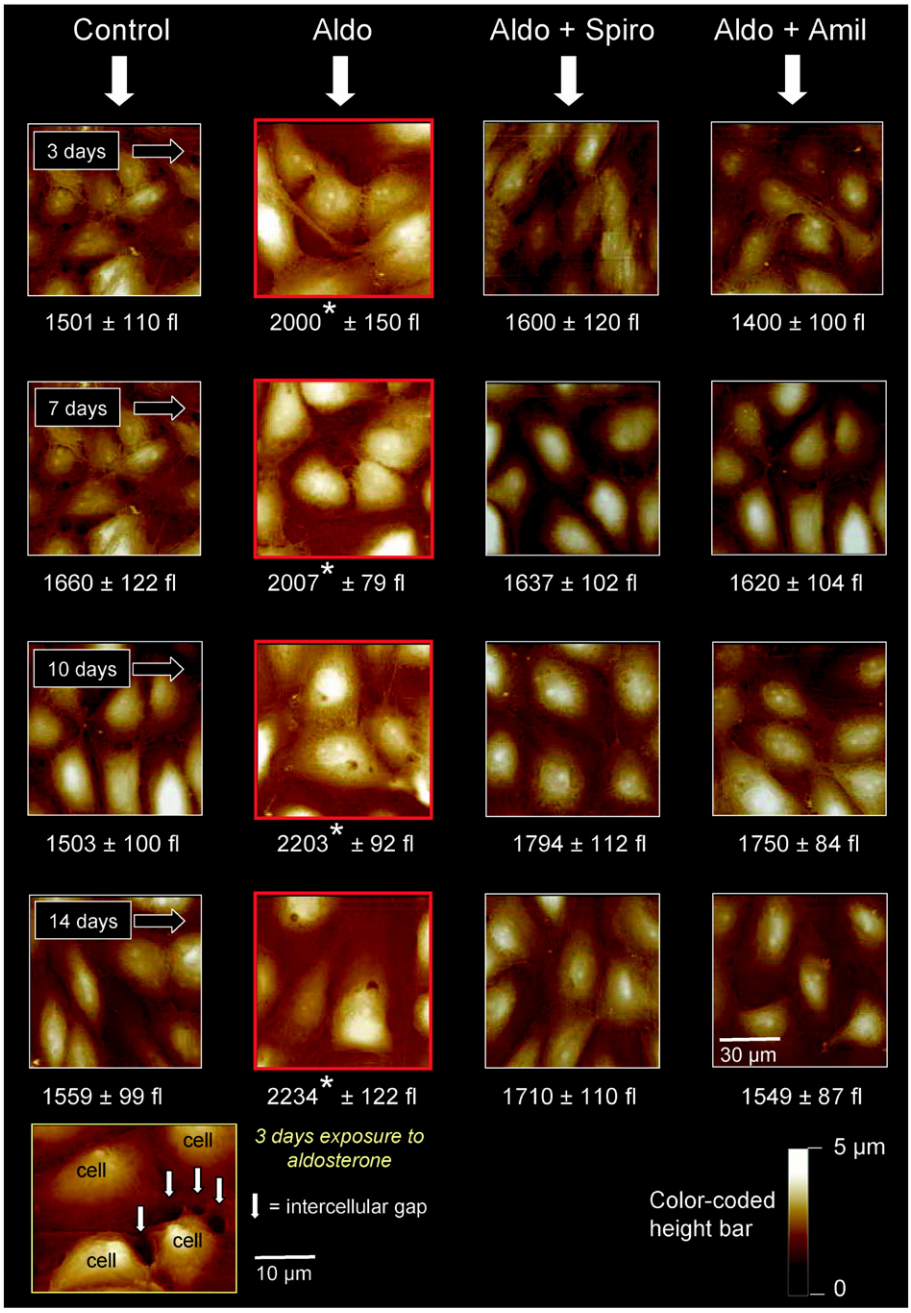

Fig. 3. Images (top views) of human umbilical venous endothelial cells obtained with atomic force microscopy. Experiments were performed in primary cultures over a period of 2 weeks. The third dimension (cell height) is color-coded (see vertical color bar at bottom). 10 nM aldosterone swells the cells (see red-framed images) but diuretics (100 nM mineralocorticoid antagonist spironolactone, coincubated with aldosterone or 1 µM plasma membrane sodium channel blocker amiloride, added 60 min prior to imaging) prevent it. Please, note intercellular gaps (bottom image) during aldosterone exposure. Numbers below the respective images represent single cell volumes in femtoliter. Asterisks (*) indicate significant differences ($p<0.05$) between aldosterone-treated endothelium and the other mean values at a given time of incubation (modified after [16]).

4. Aldosterone regulates the expression of endothelial sodium channels

Aldosterone enters the cell, binds to the cytosolic mineralocorticoid receptor (MR) and translocates into the nucleus where it triggers a signal cascade and induces the transcription of a large repertoire of aldosterone-responsive genes and a *de novo* synthesis of proteins, such as ROMK, Na^+/K^+-ATPase and ENaC [36–38]. As a result, sodium is retained and potassium is excreted by the nephron. In addition to this classical response to aldosterone it has become increasingly apparent that besides this slow (hours) genomic signalling pathway, a fast non-genomic pathway does also exist which is characterized by an early onset (sec to min) [2,3,39] and an insensitivity to transcription inhibitors [40]. Aldosterone seems to regulate ENaC via both fast and slow pathways [41]. More than 10 years ago, ENaC was identified and cloned from epithelial tissues by Canessa et al. [42]. Up to now four different subunits have been identified (α, β, γ and δ) and it was shown that they are expressed in a tissue specific manner [43–45]. The membrane insertion and activation of the sodium channel are complex and described in a number of excellent review articles [46–48]. Although, ENaC was for a long time considered to be limited to the kidney, colon and lung it is now known that vascular endothelial cells also express ENaC, in addition to mineralocorticoid

receptors [12,14,49], where it plays a crucial role in endothelial function [24,25]. It could be shown that the expression of ENaC in endothelial tissues is regulated by aldosterone [14,50].

4.1. Short-term effects of aldosterone on ENaC expression

Aldosterone activation of ENaC usually takes some hours to develop and can be blocked by inhibitors of transcription. There is increasing evidence that aldosterone additionally may have acute effects on ENaC expression. Such rapid effects have been described in kidney and colon [51–53]. Furthermore, it was demonstrated that in principal cells of the renal cortical collecting duct aldosterone modifies the rapid surface expression and insertion of ENaC via a protein kinase D dependent mechanism [54]. In this context, a membrane receptor was detected which could mediate the aldosterone effects and enables the rapid insertion of ENaC molecules into the plasma membrane [55]. To explore the 'time scale' of aldosterone regulatory pathways in endothelia, short-term experiments were performed on live endothelial cells. Using an AFM, it was found that within 10 min aldosterone increases endothelial cell volume by approximately 20%. Since this effect can be prevented by the highly specific ENaC blocker amiloride and the mineralocorticoid receptor antagonist spironolactone, it was concluded that this rapid response to aldosterone is mediated by ENaC [26]. Furthermore, it was tested whether the aldosterone-induced sodium influx rapidly modifies the biomechanical properties of the cells. Human endothelium was treated with physiological concentrations of aldosterone to stimulate ENaC expression and membrane insertion. Interestingly, within 5 min the application of amiloride decreased the surface area by almost 45% (Fig. 4) [50]. This intriguing finding supported other experiments where a fast amiloride-induced cell shrinkage in aldosterone-pretreated cells was detected [11]. It is possible that the reduction of the intracellular sodium concentration, due to the functional blockade of ENaC, induces a signalling cascade which initiates the degradation of the channel.

It is suggested that aldosterone inserts, as a non-genomic short-term response, preformed ENaC molecules into the plasma membrane of endothelial cells which activate sodium and water influx into the cell. As a result, the volume and surface of the cells transiently increase.

4.2. Long-term effects of aldosterone on ENaC expression

As described above, aldosterone induces sustained changes in the morphology of endothelial cells. Since this can be blocked by amiloride, it can be suggested that the endothelial ENaC is involved in this cellular event. With semi-quantitative RT-PCR, we have demonstrated that the mRNA expression level of ENaC in human endothelial cells is regulated by aldosterone. Incubation in 1 nM aldosterone increases the α-ENaC subunit mRNA in human endothelial cells by approximately 22%, while co-incubation with 100 nM spironolactone, a mineralocorticoid receptor antagonist, completely prevents this effect (Fig. 5). This indicates that aldosterone activates the transcription of ENaC via the (slow) genomic pathway thereby effectively increasing the total amount of proteins in the cell. By using the Western blot technique, it could be confirmed that long-term incubation with aldosterone increases the total ENaC protein amount by approximately 50%, which also can be reduced by spironolactone [14]. This, as discussed above, may contribute to the rise in cell mass.

Accordingly, in immunofluorescence experiments, aldosterone stimulates the insertion of ENaC molecules into the apical membrane by approximately 30% within 72 h, spironolactone again can prevent the effect (Fig. 6) [50]. Taken together, these findings demonstrate that long-term aldosterone treatment of endothelial cells leads to an augmented synthesis and membrane abundance of ENaC molecules. It has been described that the C-terminus of the α-ENaC subunit interacts with F-actin in the submembraneous cytoskeleton [56,57] which seems to be important for the proper function of endothelial cells [12]. By increasing the number of ENaC molecules in the plasma membrane, the interaction with proteins of the cortical cytoskeleton could be strengthened leading to an increased mechanical stiffness of the cells.

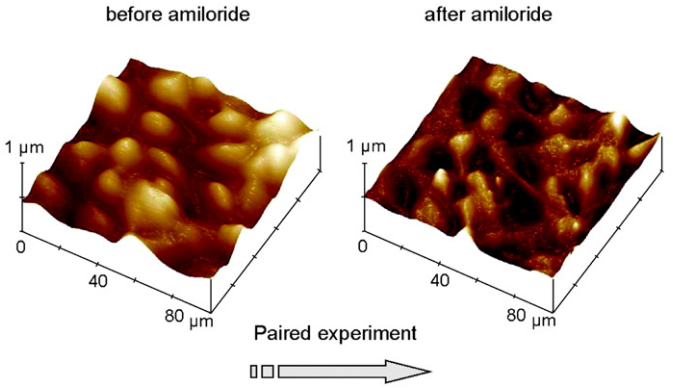

Fig. 4. Human endothelial cells were maintained for 72 h in aldosterone-containing medium. The paired experiment shows individual cells before amiloride application (left image) and 5 min after amiloride exposure (right image). Cells shrink in volume and decrease in apical surface by about 45% when 1 µM amiloride is added. (modified after [50]).

5. Sodium modifies the properties of endothelial cells

As described above the highly specific and functional ENaC blocker, amiloride has the ability to prevent cell swelling [26] and surface area increase [50] and to reduce the stiffness of endothelial cells [23]. This suggests that the sodium influx into the cell is a key player in these events. Recently, it was shown that plasma sodium concentrations above 140 mM (physiological concentration) stiffen endothelial cells and reduce the release of NO [24]. Since NO is the major regulator of blood vessel tone, this inverse correlation between stiffness and NO release may be important both physiologically and medically. It implies that the concentration of plasma sodium determines endothelial cell elasticity, and that the vascular

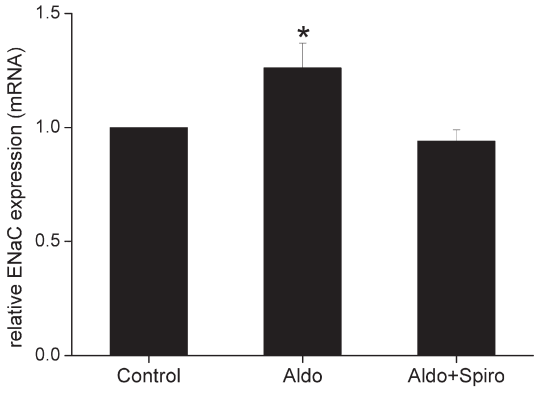

Fig. 5. With semi-quantitative RT-PCR, the relative expression levels of α-ENaC mRNA isolated from human endothelial cells (HUVEC) were detected. Control = HUVEC incubated with solvent; Aldo = HUVEC incubated with 10 nM aldosterone for 72 h; Aldo + Spiro = co-incubation with 10 nM aldosterone and 100 nM spironolatone for 72 h. Aldosterone significantly increased (*) the transcription of α-ENaC compared to control conditions while spironolactone prevented this effect ($n = 3$).

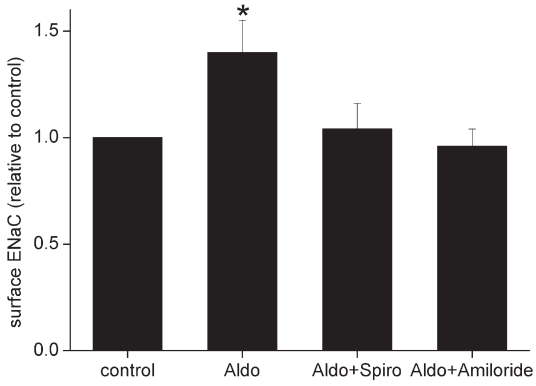

Fig. 6. The relative surface expression of ENaC molecules was detected with specific anti α-ENaC antibodies. Control = HUVEC incubated with solvent; Aldo = HUVEC incubated with 10 nM aldosterone for 72 h; Aldo + Spiro = co-incubation with 10 nM aldosterone and 100 nM spironolatone for 72 h; Aldo + Amiloride = aldosterone-pretreated cells were incubated with 1 μM amiloride for 1 h. Mean values of 10 cells per group are given. The asterisk indicates the significance difference in comparison with control ($p<0.01$). (modified after [50]).

endothelium may therefore participate in sodium-mediated changes in blood vessel function [24]. It appears therefore that sodium *per se* might be a major regulator of these cellular events and possess similar characteristics to those of aldosterone. Excessive salt consumption leads to the development of hypertension and cardiovascular disease [58] and a high salt intake increases local aldosterone production in cardiovascular tissue [59]. Therefore, high extracellular sodium could lead to the following sequence of events: Due to an increase of extracellular sodium, aldosterone is locally produced in endothelial tissues and stimulates the fast membrane insertion of ENaC molecules which, after some delay, increase the stiffness of the cells. If aldosterone is raised over a prolonged time, the stiff endothelial cells release less nitric oxide, which is perhaps one of the abnormalities which cause progressive vascular dysfunction.

6. Regulation of nitric oxide release by aldosterone

The signaling molecule NO is a gaseous radical with a relative short half life of less than 5 s and a very short diffusion distance of ~300 μm [60,61]. It is the major regulator of the vascular smooth muscle tone. NO is synthesized in the vascular system by the endothelial nitric oxide synthase (eNOS). In addition, two other isoforms of the nitric oxide synthase (NOS) are known, neuronal and inducible NOS (nNOS, iNOS), which in the healthy organism play a minor role in the regulation of endothelial function. Upon synthesis in the endothelium, NO diffuses the short distance to the adjacent vascular smooth muscle cells. There it activates soluble guanylylcyclase (sGC), which, via cGMP, phosphorylates the myosin light chain kinase and Ca^{2+}-ATPase, thereby inducing vasodilation. Additionally, to its function in blood pressure control NO also plays a role in other signaling pathways.

The regulation of eNOS is rather complex and the exact mechanisms are not yet completely understood [62,63]. In the vascular system, aldosterone is an important regulator of NO bioavailability. Since it acts via a fast (min) non-genomic pathway, as well as via a slow (hours) genomic pathway, the changes in cellular function it induces can be observed at different time scales. Some of these responses are listed in Tables 1 and 2.

6.1. Short-term effects of aldosterone on nitric oxide release

The acute effects of aldosterone on NO release are controversial (see Tables 1 and 2). In the literature, experiments can be found which indicate a short-term increase in NO bioavailability whereas other experiments apparently show the opposite. One of the sources of this discrepancy may be the variable definition of 'short-term' and 'long-term'. In some publications, a pre-incubation of aldosterone for more than 1 h is still entitled 'short-term' or 'acute' whereas others consider seconds and minutes as 'acute.' For the sake of clarity, only aldosterone-induced effects over periods of up to 30 min will be referred to as 'short-term.' Other experiment with in which aldosterone application times are longer than 30 min will be regarded as 'long-term.' Nevertheless some controversies remain as indicated in Tables 1 and 2.

Publications report that acute aldosterone treatment inhibits endothelial NO release. Aldosterone induces a calcium-dependent vasoconstriction via the activation of protein kinase C (PKC) [64,65]. This effect can be mimicked by the application of the eNOS blocker *N*-Nitro-L-arginine- methyl ester (L-NAME) which indicates that aldosterone inhibits eNOS activity at an early stage.

Table 1
Short-term effects of aldosterone on endothelial NO release.

[Aldosterone] in medium or infusion	In vivo/in vitro/in situ	NO bioavailability, ↑ / ↓ / ↔	Method	Ref.
3.3–55 pM	In vivo	↑	FBF	[94]
0.01 nM–1.0 μM	In vitro	↑	Fluorescent probe	[70,76]
5–10 nM	In vitro	↑	NO selective electrode, fluorescent probe	Fels et al. (unpublished)
≤1 nM	In situ	↑	Diameter of arterioles	[82]
18 nM_{pl}	In vivo	↑	FBF	[71]
0.01–100 nM	In situ	↑	Diameter/ constriction of arteries	[69]
0.1–10 nM	In situ	↓	Diameter/ constriction of arteries	[64,95]
2.5 pM/min	In vivo	↓	FBF	[96]
1.3–3.3 nM_{pl}	In vivo	↓	FBF	[97]
5.3–20.5 nM	In vivo	↓	Systemic vascular resistance	[98]
28–277 nmol/ min	In vivo	↔	FBF	[99]
1.4 μmol injection	In vivo	↔	Renal plasma flow	[100]

Conditions of aldosterone treatment and effects on endothelial NO release are summarized (↑ = increase, ↓ = decrease, ↔ = no change detected, n.m. = not measured, FBF = forearm blood flow).

Table 2
Long-term effects of aldosterone on endothelial NO release.

[Aldosterone] in medium or infusion	In vivo/in vitro/in situ	NO release, ↑ / ↓ / ↔	Method	Ref.
788 nM/d fludrocortisone	In vivo	↑	FBF	[94]
0.45 nM	In vitro	↓	Nitrite concentration	[24,25]
100 nM	In vitro	↓	cGMP measurement	[29]
Only endogenous	In vivo, hypertensive rats	↓	eNOS protein level, eNOS phosphorylation, nitrite concentration	[81]
2 μmol/h	In situ, rat mesenteric arteries	↓	Vessel relaxation studies	[77]
2 μmol/h	In vivo	↓	Systolic blood pressure analysis	[78]
Only endogenous	In vivo	↓	FMD	[28]
12 pmol/min/kg	In vivo	↓	FBF	[80]
n.m.	In vivo	↓	FBF	[79]

Conditions of aldosterone treatment and effects on endothelial NO release are summarized (↑ = increase, ↓ = decrease, ↔ = no change detected, n.m. = not measured, FBF = forearm blood flow, FMD = flow mediated dilation).

Additionally, Michea et al. showed that the cytosolic mineralocorticoid receptor (MR) must be the mediator of this response by reason that the aldosterone receptor antagonist eplerenone inhibits the negative effect of acute aldosterone treatment, [65]. Furthermore, endothelial Na^+ transport seems to be involved in aldosterone-dependent acute eNOS inhibition. Application of high concentrations of amiloride (100 μM) which block Na^+ transport via, e.g., both the Na^+/H^+ exchanger 1 (NHE1) and ENaC inhibits the aldosterone-induced decrease of NO bioavailability [65]. However, these findings do not necessarily lead to the conclusion that aldosterone acutely inhibits endothelial NO production because rather high concentrations of sodium were used in this study [65]. Since it has been shown that chronic aldosterone treatment, together with high sodium concentrations, leads to reduced NO release in endothelial cells [24], it cannot be excluded that high sodium concentrations *per se* have induced this effect [66–68]. Additionally, it cannot be excluded that the vasoconstrictive effect of aldosterone could be independent of endothelial cells. It is likely that this effect is caused directly by an influence of aldosterone on vascular smooth muscle cells (VSMC). This view is supported by the fact that arterioles lacking the endothelium show an increased aldosterone-induced contraction compared to intact arterioles [64,69,70]. In addition, it has been shown that the co-infusion of aldosterone and L-NAME induces an increased vasoconstriction, as compared to an infusion with aldosterone alone [71]. Thus, it may be concluded that aldosterone-induced vasoconstriction detected either *in situ* or *in vivo* is not correlated with an acute inhibition of eNOS but explained by a direct aldosterone-induced contraction of smooth muscle.

This hypothesis may also explain why others reported that aldosterone induces an acute transient increase of NO bioavailability in endothelial cells. It has been shown in the afferent arterioles of renal glomeruli that the acute application of very low concentrations of aldosterone decreases the efficiency with which K^+ induces vasoconstriction [72,73]. Very low aldosterone concentration may act specifically only on the endothelial cell layer while vascular smooth muscle cells remain unaffected. It is tempting to suggest that the negative effects of aldosterone are only induced when aldosterone concentrations are high.

The assumption that aldosterone-triggered NO release occurs is supported by data from recent *in vitro* experiments. Fluorescent indicators allow the detection of NO release at single cell level. Diamonofluoresceine and its derivates (e.g., DAF-2 DA, DAF-FM DA) are commonly used for intracellular NO fluorescent imaging [74,75]. Acute addition of aldosterone leads to a transient increase of intracellular DAF-2 fluorescence intensity in primary bovine aortic endothelium [70]. Interestingly, this study revealed a dose-dependent aldosterone effect. Additionally, Mutho et al. showed that in primary bovine endothelial cells aldosterone increases eNOS phosphorylation [76]. They also showed that aldosterone-influenced NO release and that a short-term incubation of aldosterone increased eNOS phosphorylation and NO release, whereas this effect was not detected during a long-term application of aldosterone. The transient nature of NO production may be due to an acute increase in endothelial calcium for endothelial cells acutely respond to aldosterone with increasing intracellular Ca^{2+} [31,66]. Since eNOS is activated by Ca^{2+}/calmodulin, the released Ca^{2+} may potentiate eNOS activity.

We have studied the acute and chronic effects of aldosterone on the release of NO from endothelial cells. NO selective electrodes and fluorescent NO indicators were used to analyze the acute effect of aldosterone. In addition, the long-term effect of aldosterone on NO release was examined via the Griess assay. As shown in Fig. 7, an acute application of aldosterone on bovine aortic endothelial cells (GM7373) induces a transient release of NO within 20 min, whereas prolonged treatment with aldosterone decreases NO bioavailability (Fig. 8).

6.2. Long-term effects of aldosterone on nitric oxide release

In general, prolonged exposure of endothelial cells to aldosterone decreases NO bioavailability. Although a direct measurement of NO in long-term experiments is difficult (because of the short half-life of NO), there are several methods available which can detect NO metabolites, or stable NO substrates (e.g., nitrite, nitrate or sGC/cGMP). Therefore, the long-term effects of aldosterone are well documented.

As shown in Fig. 8, *in vitro* studies reveal that a prolonged exposure to aldosterone decreases nitrite concentration in the medium [25]. The concentration of cellular cGMP is reduced in parallel [29]. In addition, it has been shown that chronic aldosterone treatment induces a decreased ability of rat mesenteric arteries to contract [77]. In each of these three studies, aldosterone receptor antagonists (spironolactone, eplerenone) abolished the aldosterone-effect, which indicates that the mineralocorticoid receptor mediates the aldosterone-induced long-term decrease in NO bioavailability. This conclusion is supported by *in vivo* experiments analyzing rat arterial blood pressure [78], human forearm blood flow [28] and flow mediated dilatation, after prolonged exposure to aldosterone [79,80]. Although the specific mechanisms underlying the chronic (and pathological) aldosterone effects are still unknown, it is quite clear that aldosterone induces a decrease in eNOS expression, eNOS phosphorylation and nitrite production [81].

6.3. Acute aldosterone-induced NO release: a hypothesis

The described findings lead to a new hypothesis to explain acute aldosterone-induced NO release from endothelial cells. Controversial results obtained from different laboratories had led to a dilemma which was probably due to three facts: (i) An inconsistent definition of the terms ‘acute’ and ‘chronic.’ Therefore, it is important to have a close look at ‘time scales’ and results obtained by acute aldosterone administration should be separated from those obtained with long-term aldosterone incubation. (ii) The different aldosterone and salt concentrations used in the various studies can cause conflicting effects. High concentrations of aldosterone and/or salt may have different effects than low concentrations. Therefore, it is necessary, to take the right ‘quantities’ into account. (iii) The effects of aldosterone itself on vascular smooth muscle cells. Since endothelial cells are also targets for aldosterone, their potential response should not be neglected in both *in situ* and *in vivo* studies.

In our postulated model in endothelial cells, aldosterone inhibits NO release during long-term application whereas an acute exposure induces NO production. This short-term NO release is of transient nature with a bell-shaped dose-dependency [70,82].

This view is also supported by studies demonstrating a correlation between NO and the mechanical stiffness of the cells [24,25,83,84]. Namely, aldosterone induces a transient softening of the cells and as a result a fast and transient NO production which is reversed within minutes (Fig. 1).

The limited amount of NO produced, due to transient eNOS activity during the early response to aldosterone may not be sufficient to induce smooth muscle relaxation but, instead, it may influence other signaling pathways in the endothelial cells. It is known that, besides its function as a vasodilator, NO acts as a messenger molecule. Thus, NO can influence other signal cascades by modifying, e.g., kinases and G protein-coupled receptors [85–87]. In this context, it has been shown that shear stress-induced NO release leads to an s-nitrosylation of several proteins including ER-ATPase, HSP90, and tubulin beta chain [88]. So far, we are not aware of any study concerning aldosterone-induced protein modification by s-nitrosylation. It has already been shown that the two eNOS pools, one underneath the plasma membrane and the other in the Golgi-apparatus, are regulated separately [87,89]. Therefore, it is suggested that Golgi-linked eNOS

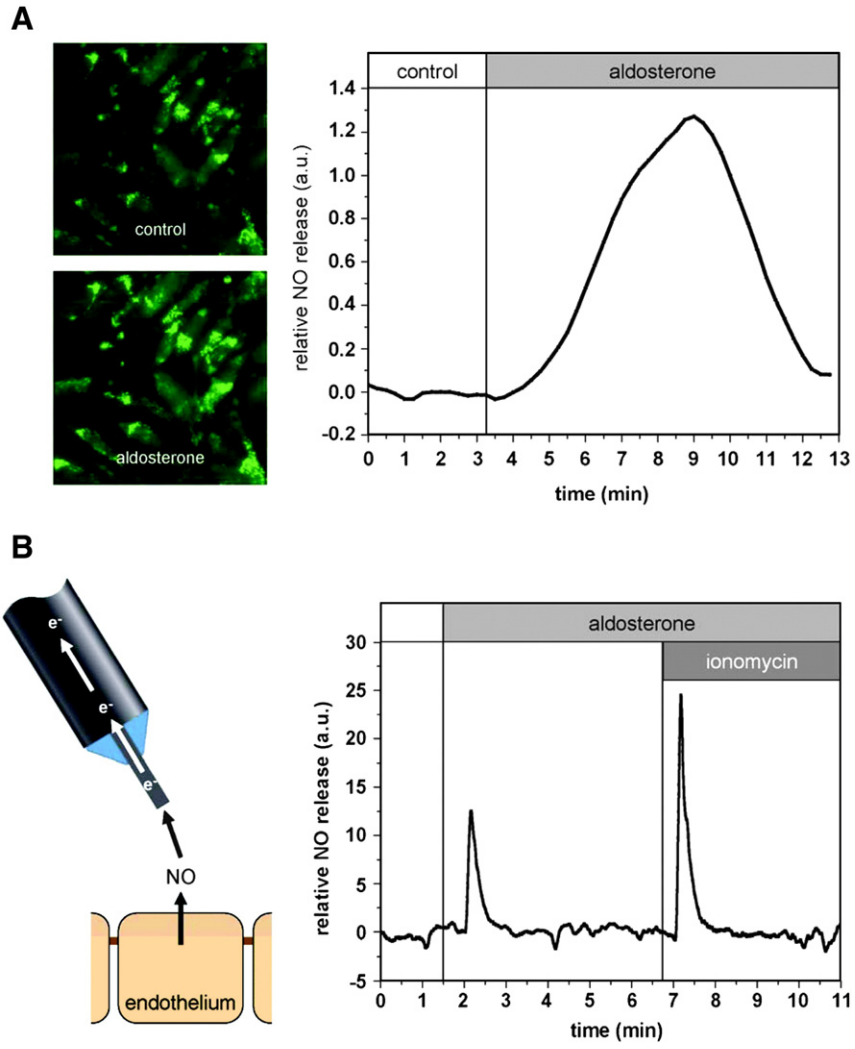

Fig. 7. Short-term effect of aldosterone on endothelial NO release of bovine aortic endothelial cells GM7373 was recorded by fluorescence microscopy or amperometric NO measurement. (A) Cells were loaded with the intracellular NO indicator DAF-2 DA (Merck, Darmstadt, Germany). Due to the covalent binding of NO to the fluorescent indicator, the slope of the measured fluorescence intensity was calculated and analyzed as relative NO release. (B) NO release into the culture medium was detected with the NO selective electrodes (ISONOP007, WPI, Serasota, USA). Upon application of aldosterone, a rapid and transient increase of NO synthesis was detected. Ionomycin was used as a gold standard for quantification during the amperometric NO detection (Fels et al., unpublished).

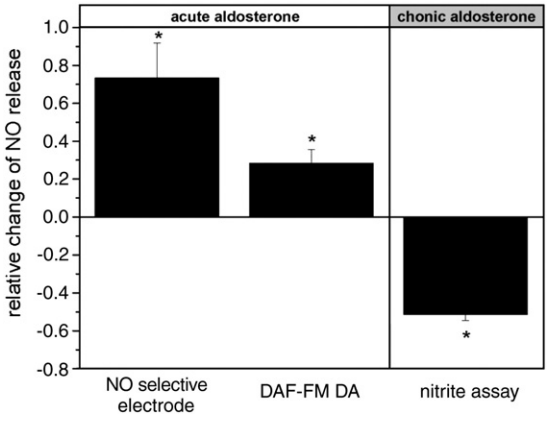

Fig. 8. Acute and chronic effects of aldosterone on NO release by bovine aortic endothelial cells are shown. Short-term effects were analyzed with NO selective electrodes (ISONOP007, WPI, Serasota, USA) ($n=5$) and the fluorescent NO indicator DAF-2 DA (Merck, Darmstadt, Germany) ($n=40$). Nitrite assays were used to document effects of aldosterone in a period of 24 h ($n=16$). Each experiment was significantly different to the respective control ($^*p \leq 0.05$) (modified after [25], Fels et al., unpublished).

has a higher affinity to aldosterone than eNOS underneath the plasma membrane. This suggestion is also supported by several other publications, which investigated eNOS activity at the Golgi-apparatus and the plasma membrane [90–93].

7. Conclusions

The mineralocorticoid hormone aldosterone is a critical regulator of the biomechanical properties of endothelial cells, and its action is mediated by endothelial ENaC. The mechanisms involved are either 'fast' (minutes) or 'slow' (hours). The latter occurs when the hormone is released after a stimulus from the adrenal cortex and acts 'systemically.' Both endothelial and vascular smooth muscle cells are targeted by aldosterone, resulting in blood vessel constriction.

Nevertheless, what are the physiological mechanisms responsible for the short-term effects? A low concentration of aldosterone stimulates the release of NO within minutes paralleled by a transient softening of endothelial cells. Such 'local' transient aldosterone-induced NO release should relax smooth muscle but this tends to be opposed by the direct constrictive effect of 'systemic' aldosterone on

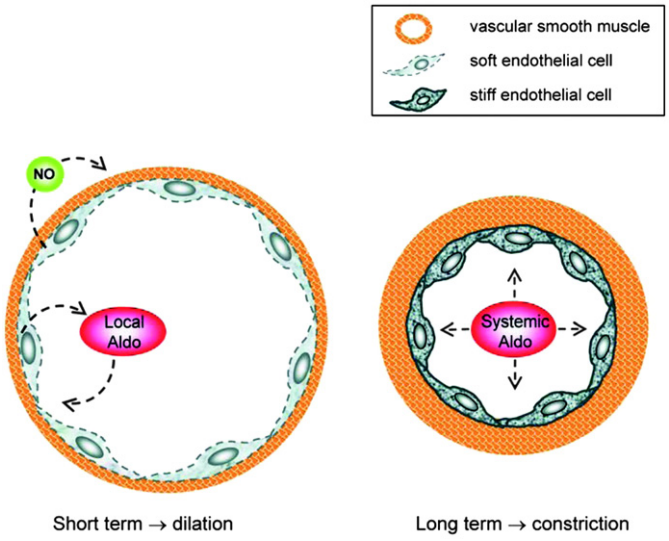

Fig. 9. Left side: Local endogenous aldosterone production stimulates the release of NO which acts directly on the smooth muscle cells. This leads to transient short-term vasodilation. Right side: Systemic aldosterone reduces the NO release leading to long-term vasoconstriction.

vascular smooth muscle (Fig. 9). Thus, the net result (constriction or dilation) appears to be predominantly dependent upon both systemically and locally released aldosterone. The subject needs further investigation.

Acknowledgements

Work in the authors' laboratory was supported by grants from the Deutsche Forschungsgemeinschaft (OB 63/17-1 and Koselleck Grant OB 63/18). We are grateful to Prof. Hugh de Wardener from Imperial College, London for suggestions to the manuscript, and to Prof. Gerhard Giebisch from Yale University Medical School who has supported our work over many years. The works of Ms. Xenia Husser for RT-PCR experiments and Ms. Marianne Wilhelmi for nitrite measurements are gratefully acknowledged.

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